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Unbiased, high-throughput identification of T cell epitopes by ELISPOT

Paul V. Lehmann[1], Diana R. Roen[1], and Alexander A. Lehmann[1]

[1]Research & Development Department, Cellular Technology Limited, Shaker Heights, OH, 44122, USA

† Correspondence author: Paul V. Lehmann

Paul V. Lehmann

Research & Development Department,

Cellular Technology Limited,

20521 Chagrin Blvd, Shaker Heights, OH 44122

EM: paul.lehmann@immunospot.com

Running Head: T cell epitope mapping

Abstract

Recent systematic immune monitoring efforts suggest that, in humans, epitope recognition by T cells is far more complex than has been assumed based upon minimalistic murine models. The increased complexity is due to the higher number of HLA loci in humans, the typical heterozygosity for these loci in the outbred population, and the high number of peptides that each HLA restriction element can bind with an affinity that suffices for antigen presentation. The sizable array of potential epitopes on any given antigen is, due to each individual's unique HLA allele makeup. Of this individualized potential epitope space, chance events occurring in the course of the T cell response determine which epitopes induce dominant T cell expansions. Establishing the actually-engaged T cell repertoire in each human subject, including the individualized peptides targeted, therefore requires the systematic testing of all peptides that constitute the potential epitope space in that person. The goal of comprehensive, high-throughput epitope mapping can be readily established by the methods described in this chapter.

Key words: Elispot, Fluorospot, CD4 T cell, CD8 T cell, epitope prediction, T cell determinant, immune monitoring.

1. Introduction

The B and T lymphocyte systems have evolved to cooperate in highly specific antigen recognition. To do so, B cells and T cells rely on two fundamentally different criteria for telling antigens apart among the millions of structures foreign to the body to which they must respond on demand, and a similar number of self-antigens that they must neglect [1]. B cells assess the form and the 3-dimensional shape of native antigens: B cell receptors (BCR) bind to complimentary surfaces on the antigen, small areas called B cell determinants; however, they do so without discriminating the antigen's chemical nature as a protein, sugar, nucleic acid, etc. T cells, in contrast, recognize proteins only, discriminating between them solely based upon their unique amino acid sequence. For this to occur, short peptide fragments of the protein need to bind to specialized antigen-presenting molecules, so-called major histocompatibility complex (MHC) molecules. The antigen-derived peptide is accommodated in the "peptide-binding groove" of these MHC molecules (which are called HLA molecules in humans), whereby the unique peptide binding motif of each MHC/HLA allele defines which peptide is bound, and which is not. The T cell antigen receptor (TCR) binds this binary structure consisting of the nominal peptide fragment of the antigen aligned on an MHC/HLA molecule for antigen recognition. B cell responses that lead to production of IgG, IgA or IgE antibodies strictly depend on T cell help, that is, the verification of the antigen's identity via its amino acid sequence by T cells.

The precise identification of an antigen via its amino acid sequence is at the heart of self/non-self-discrimination by T cells, there has been a strong evolutionary pressure to optimize this strategy [2]. Subsequently, the antigen presenting MHC/HLA molecule system evolved to be polygenic (there are three major Class I and three Class II loci for humans) in addition to being one of the most polymorphic gene systems known. The human genome contains hundreds of alleles for

these loci, whereby each of the alleles encode MHC/HLA molecules that differ in their peptide binding groove, affording each of them a unique peptide binding motif and thus peptide presentation property. While certain HLA alleles are more frequent in some human populations than in others, for example the A2 allele in Caucasians, even within the A2 positive subpopulation there are hardly two individuals who would also match in the other five Class I alleles they express, and consequentially, there are hardly two humans on this planet who would present the identical set of peptides for their T cells to recognize. The MHC/HLA system has evolved to make antigen recognition unique in each individual, thereby minimizing dangerous consequences of failures in self/non-self-recognition. By mutation, pathogens could easily evade T cells if the potential epitope space was limited in individuals. This danger is minimized for the individual by the presence of (in the typical case of heterozygosity for all three loci) six HLA Class I, and six Class II molecules, each with different peptides binding motifs. And even if the unique HLA molecule set expressed by a certain individual does not convey T cell high responder status to an antigen, such can endanger only that subject, but not other individuals who express different sets of HLA molecules. Due to HLA polygenism and polymorphism, the array of antigenic peptides presented to T cells is highly diverse across populations and each individual has a unique potential epitope space.

The peptide binding motifs for the individual allelic HLA molecules are increasingly well defined, and it is now possible to predict *in silico* the peptides of an antigen that will be presented by this HLA molecule [3]. Individuals who share an HLA allele can be expected to share a fraction of their potential epitope space (but will differ in the potential epitope space defined by all the other, non-overlapping HLA alleles expressed in the individual). Subsequently, one might expect that individuals who share an HLA allele will predictably respond to an overlapping set of peptides. This notion, originating in reductionist studies of simple model antigens in inbred mice (expressing minimal MHC diversity) does not even hold up for F1 mice: while in such mice the

same set of peptides is selected for antigen presentation due to their shared MHC makeup, their T cells typically show individual, unpredictable “aleatory” response patterns to these peptides [4]. Aleatory recognition likely results from the low frequency of antigen-reactive T cells being linked to fate decisions dictated by chance events [5-7]. Systematic epitope mapping showed that also in humans aleatory epitope recognition by T cells prevails, even in HLA-A2 allele-matched subjects, resulting in highly divergent and individualized T cell response patterns [8,9]; see also Figure 1. Therefore, while the potential epitope space of an individual can be predicted *in silico,* based on the peptide binding motives of the allelic HLA molecules present in an individual, the minor subset of such peptides that actually triggers a dominant T cell response cannot be predicted. The latter constitutes the individual’s expressed epitope space, being a minor fraction of the individual’s potential epitope space.

The expressed epitope space consists of the individual peptides of an antigen that the T cell system actually targets in an individual (highlighted in color in Figure 1). The number of such peptides depends on the size of the protein. The number (frequency) of T cells present in the blood that are specific for a given peptide gives insights into the extent of clonal expansions that the cells with this peptide specificity have undergone during the immune response. Within an individual, different peptides of the antigen can elicit strong, medium, or weak T cell expansions, to which we refer as dominant, subdominant, or cryptic epitopes (see the red, orange, and yellow highlights in Figure 1). The total number of T cells recognizing all expressed epitopes in an individual defines the magnitude of the T cell immunity against the antigen. The cytokine signature of these antigen-specific T cells provides insights about the quality of T cell-mediated immunity that becomes shaped by an instructed differentiation process in the course of the immune response resulting in the generation of T cells with different effector functions [10].

T cell immune monitoring aims at assessing the magnitude and quality of the antigen-specific T cell compartment in an individual. Its successful implementation depends on covering the entire expressed epitope space of each individual. Confining T cell immune monitoring to select peptides only, as it has been frequently done, e.g., to one or a few *in silico* predicted, or previously established epitopes, runs great risk of underrepresenting – or even missing-- the antigen-specific T cell pool (Figure 1). The peptide(s) chosen might be just one of several dominant epitopes recognized in that individual, or, worse, might be recognized in a subdominant, or cryptic fashion, or not at all, while other peptides dominate. One theoretical solution would be to narrow in on testing all peptides that encompass the potential epitope space for an individual [11]. This would require making *in silico* predictions for all HLA alleles expressed in each individual, and custom preparation of matching peptide libraries for each subject. Recent studies suggest, however, that it is not necessarily the peptides with highest predicted binding scores that constitute the actually recognized epitopes, but rather that immune dominant epitopes frequently rank downstream in the predicted binding hierarchy [12,13,5,9,14] – see also in Figure 1 the predicted peptides (highlighted in green) vs. those that actually elicited CD8+ T cell responses. Thus, *in silico* prediction-based T cell monitoring would need to accommodate an estimated 30 peptides with the highest binding scores per HLA allele expressed in each test subject, i.e., up to 180 customized peptides per individual for CD8+ T cell detection alone, a scope that is hardly realizable for immune monitoring purposes. Therefore, T cell immune monitoring is increasingly moving towards “agnostic testing”, an approach in which all possible peptide segments of the antigen are systematically covered (Note 1). Agnostic testing can be done, depending upon the size of the antigen, by using several peptide pools, each of which containing up to 200 individual consecutive peptides [11]. This so called “mega peptide pool” approach has the advantage of practicability as it is economic on white blood cell numbers needed and labor involved. Its disadvantage is that the many peptides compete with each other for HLA-binding whereby irrelevant (non-recognized,

but HLA-binding) peptides can outcompete the binding of the relevant (recognized) peptides[15]. Thus, mega peptide pools are prone to under-represent the actual size of the engaged antigen-specific T cell population. An additional disadvantage of the mega peptide pool approach is its low resolution; that is, it does not provide information on the identity, diversity, and hierarchy of the individual epitopes recognized. Comprehensive epitope mapping [16], the subject of this chapter, overcomes all these limitations providing the highest possible resolution on the actual antigen-specific T cell compartment operational in a given test subject.

In the comprehensive single peptide epitope mapping approach, peptides from libraries that systematically cover the sequence of the antigen are tested individually (Figure 1). Thereby every peptide of the potential epitope space is covered, one by one. Such testing involves - depending upon the size of the antigen of interest – hundreds, or even thousands, of individual peptides. In a modification, this testing can also be done in an easy to deconvolute matrix format (Figure 2). Dependent on the configuration of the matrix, this approach cuts down substantially on the number of test conditions (Note 2), and thus on the number of cells needed, however, without losing the ability to identify the individual peptides recognized. Also, the number of peptides within a matrix is not high enough for peptide competition to be of major concern [17]. Importantly, as every possible epitope is covered, in both the single peptide or the matrix-based epitope mapping approach, testing is done “agnostically”, that is, without the need of tailoring the test peptides to the HLA type of the test subject.

Until recently, comprehensive T cell epitope mapping has been limited to short antigens that involve less than 200 individual peptides or matrix pools. Limiting factors have been the number of peripheral blood mononuclear cells (PBMC) available for testing, and lack of a technology that enables such testing in a streamlined, high-throughput manner, while doing so in a cost- and labor-efficient way. ELISPOT/FluoroSpot is such a technology (we refer to both collectively

as ImmunoSpot®, as they differ only in the enzymatic vs. fluorescent detection of the plate-bound analyte). The assay principle is shown in Figure 3. In ImmunoSpot® assays, PBMC are plated at 100,000 -1,000,000 PBMC per well into 96 well plates (Note 3), but the assay can be miniaturized by using 384 well plates [18] reducing by one third the number of PBMC and peptides needed (Note 4). Moreover, testing peptides in matrix format can cut down on the cell numbers needed. For example, 1263 peptides can be arranged into a 36 x 36 matrix generating a total of 72 peptide pools that can be tested and then deconvoluted to identify the individual peptides of interest, reducing 94% of the test conditions (Note 2). Cell numbers are therefore no longer limiting for the comprehensive testing of T cell epitope recognition of complex antigens, or even genomes. Neither is the cost and labor involved, as outlined below.

ImmunoSpot® assays measure the number of T cells in PBMC that engage in cytokine production after recognition of their cognate antigen/peptide [19]. As naïve T cells do not produce cytokine upon their first antigen encounter, and occur in very low frequencies within PBMC, only *in vivo* antigen-primed, clonally expanded memory or effector T cells are being detected in the typical recall assay format when PBMC are exposed to antigen *in vitro* for 24h [20]. As these T cells secrete the cytokine, it is captured around the cell by a cytokine-specific capture antibody – leaving a secretory foot print on the membrane that is then visualized by the addition of a cytokine-specific detection antibody (Figure 3). Typically, IFN-γ secretion by T cells is being measured, but the assay can be multiplexed to detect up to four cytokines simultaneously (Note 7) permitting to define other effector cell lineages and the "fitness" thereof [10]. The number of spots per well is then counted by a dedicated reader in a fully automated fashion, reflecting the number of peptide-specific T cells secreting the interrogated cytokine within the number of PBMC plated into that well [21]. In this way, the magnitude of the T cell population responding to each peptide is established, permitting to identify dominant, subdominant, cryptic and non-immunogenic peptides (see the color code in Figure 1). The sum

of T cells recognizing all these epitopes reflects on the total size of the antigen-specific T cell pool, i.e., the magnitude of T cell-mediated immunity [9]. Typical results of such an experiment are shown in Figure 1. If the assay was done in a multiplexed cytokine format, the quality of the antigen-specific T cell response is also revealed [10].

In the closing part of the Introduction, we are outlining the logistic how such "monstrous" tests can be performed with relatively minor effort; the specifics are provided below. The process starts with (Step 1) creating the plate layout, assigning in successive order a consecutive peptide to each well (See Figure 1), whereby wells A1 to A4 on each plate are typically reserved for positive and negative controls, respectively (Note 8). A dedicated software is available for creating such plate layouts (SpotMap™ by CTL) that also includes barcode printing options for the plates (Note 9), and provides the template for the subsequent automated analysis of the plate so that "spot forming unit" (SFU) counts for each well on each plate can be automatically assigned to the individual peptide's (or array's) identity for each test subject. In Step 2, this plate layout is provided to a peptide manufacturer to synthetize the peptide library according to this design into barcoded 96 (or 384) well plates (Notes 10-12). Step 3 initiates with dissolving of the peptides, and diluting them into ready-to use barcode- and color-coded 10 x Master Plates to assure the peptides' identity, concentration, and purity (See Figure 4, Figure 5, and Notes 13-17). Step 4 is the actual high throughput ImmunoSpot® epitope testing, in which 96 channel (or 384 channel) pipettors are used to transfer the peptides from the 10 x peptide master plates to the test plates containing the PBMC; suggestions are made how to implement such "monstrous" experiments with relatively little labor, and, importantly, minimizing the possibility of experimental error (Notes 18-22). The final step, Step 5 is the reading and databasing of the experimental results, a fully automated process [11] (Note 23).

2. Materials

Thawing of Cryopreserved PBMC

1. Cryopreserved PBMC sample (Note 24)
2. DNase-containing washing medium (Note 25)
3. CTL-Test™ Medium (Note 22)
4. 50 mL conical tubes
5. Parafilm foil
6. Tabletop Centrifuge
7. Hemocytometer(s)
8. CTL ImmunoSpot® Reader, any model

ImmunoSpot® Assay

1. Human IFN-γ Pre-coated ImmunoSpot® Kit, including:
    a. Pre-coated ELISPOT plate, PVDF membrane
    b. Diluents B, C, and Blue
    c. Detection Ab
    d. Streptavidin-AP
    e. Substrate solutions S1, S2, and S3
2. CTL-Test™ Medium
3. Test antigens/peptides (Notes 11-17)
4. PBS
5. PBS-T (PBS + 0.05% Tween-20)
6. Distilled water

3. Methods

Warm all media to 37°C before use. All thawing and culture steps should be performed with warm (37°C) media to retain functionality of the cells.

Perform all culture steps in a biological safety cabinet following all appropriate safety protocols.

3.1. Thaw cryopreserved PBMC (sterile conditions):

1. Place cryovials into the 37°C water (or, preferably bead) bath for 8 minutes to thaw.
2. Remove cryovials from 37°C bath and wipe with 70% ethanol before unscrewing caps.
3. Using a serological pipet, transfer contents of cryovial into a 50 mL conical tube. Up to 5 vials of the same donor can be pooled into one 50 mL tube.
4. For each cryovial used, rinse the cryovial with 1 mL Anti-Aggregate solution (Note 25). Transfer the rinse solution to the 50 mL tube slowly, dropwise, while swirling the tube to ensure adequate mixing of the cells and thawing medium.
5. Add an additional 2 mL Anti-Aggregate solution to the tube dropwise while swirling. The cells are now in a total of 4 mL.
6. Add the final 6 mL of Anti-Aggregate solution to the tube, swirling gently to mix. The cryovial is now resuspended in a total of 10 mL of Anti-Aggregate solution. (If additional cryovials are pooled, calculate using 1 mL cell suspension + 9 mL Anti-Aggregate solution for each cryovial to find total resuspension volume).
7. Cap the 50 mL conical tube tightly and invert twice to mix.
8. Centrifuge PBMC at 300 x g for 10 minutes at RT.
9. Discard supernatant and flick the bottom of the conical tube gently to resuspend the cell pellet. Add 10 mL Anti-Aggregate solution for each cryovial thawed. Cap the tube and invert gently twice to mix.
10. Pipet 20 µL Live/Dead Cell Counting dye (e.g., acridine orange/propidium iodide) onto a small piece of parafilm.

11. Remove 20 μL of cell suspension and add to the Live/Dead Cell Counting dye. Pipet up and down 3-5 times to mix avoiding formation of bubbles.
12. Transfer 20 μL of the cell and dye suspension into each chamber of a hemocytometer.
13. Count live cells under UV microscope, or using CTL's Live/Dead Cell Counting Suite.
14. Centrifuge the 50 mL tube containing PBMC again at 300 x g for 10 minutes at RT.
15. Discard supernatant and gently flick the bottom of the tube to resuspend the cell pellet.
16. Resuspend the cell pellet in pre-warmed (37°C) CTL-Test™ Medium to the desired concentration (Notes 3,4,26).

3.2. Plate PBMC (sterile conditions):

1. Gently swirl PBMC suspension to ensure even distribution of the cells (Note 28).
2. Using wide-orifice tips and a 96 channel pipettor, add 180 μL PBMC suspension to each well of the 96 well of the pre-coated ImmunoSpot plate (90 μL for the 384 well plate) (Note 28).
3. Store plates with cells in the incubator all until the plating of the peptides.

3.3. Plate Antigens/Peptides (sterile conditions):

1. Prepare all antigens/peptides at 10 x final concentration in Peptide Master Plates). (Notes 11-17).
2. For a 96 well plate, using a 96 channel pipettor, add 20 μL of the 10 x master peptide solution per well into the pre-coated human IFN-γ ImmunoSpot® plates containing the PBMC (10 μL per well of peptide for 384 well plates).

4. Replace the plate cover and gently tap the plate on all sides to ensure even sedimentation of the cells across the membrane (Note 27).
5. Incubate plate at 37°C supplemented with 8-9% CO_2 for 24 hours. Do not disturb or relocate the plate during the incubation, as this will result in poor spot morphology. Open and close the incubator door gently to avoid disturbing the ELISPOT plate.

3.4. Develop the ELISPOT plate (non-sterile conditions):

1. Upon completion of the cell culture incubation, remove plate from the incubator. Decant the plate (or harvest the cells for additional downstream analysis, if desired; see Note 21) and wash 5 x with 200 µL PBS/well for 96 well plates (100 µL for 384 well plates) using an automated plate washer with adjusted pin height (Note 29).
2. Decant plate and wash 5 x with 200 µL PBS-T/well for 96 well plates (100 µL/well for 384 well plates).
3. Prepare Detection Solution by adding 40 µL Detection antibody to 10 mL Diluent B.
4. Decant wash from plate and add 80 µL/well Detection Solution.
5. Incubate for 2 hours at RT in the dark.
6. Decant Detection Solution and wash plate 5 x with 200 µL/well PBS-T for 96 well plates (100 µL for 384 well plates).
7. Prepare Tertiary Solution by adding 10 µL Streptavidin-AP to 10 mL Diluent C.
8. Decant wash from plate and add 80 µL/well Tertiary Solution.
9. Incubate for 30 mins at RT in the dark.
10. Decant Tertiary Solution and wash plate 5 x with 200 µL/well PBS-T for 96 well plates (100 µL for 384 well plates).

11. Decant and wash 5 x with 200 μL/well distilled water for 96 well plates (100 μL for 384 well plates).
12. Prepare Substrate Solution by adding 160 μL of S1 to 10 mL Diluent Blue. Mix well. Add 160 μL of S2 to the solution and mix well. Finally, add 92 μL of S3 to the Substrate Solution and mix well (protect from light and prepare the Substrate Solution immediately prior to use).
13. Decant wash and add 80 μL/well Substrate Solution.
14. Incubate for 15 minutes at RT.
15. Remove underdrain and rinse both sides of plates 3 x with tap water. Allow plates to dry completely prior to imaging and analysis (Note 23).

4. Notes

1. The traditional approach is to use 15-mer peptide libraries that walk the sequence with several (typically four) amino acid steps, and presently most commercially available peptide libraries follow this design [11]. While this approach cuts down on the number of peptides needed for testing, it is presently unclear how well 15-mer peptides are suited for CD8+ T cell immune monitoring (9-mers are ideal for binding to MHC/HLA Class I molecules [22]. Also, Class I molecules being closed on both ends are intolerant to frame shifts in the amino acid sequence of peptides [22] and therefore it can be expected that major gaps in epitope coverage arise with the 15-mer, 4 amino acid gap coverage).

2. Dependent on the configuration of the matrix, this approach cuts down substantially on the number of test conditions. For example, when testing a large virus like SARS-Cov-2 we identified 1263 peptides of interest. In a traditional 96 well format, 383,700,000 cells would be

needed to test these 1263 individual peptides at 300,000 cells per well. However, by generating a 36x36 matrix and running the assay in a 384 well at 33,333 cells per well, only 2,533,308 cells would be needed. Running the assay in the 384 well Matrix format requires only 0.66% of the cell material compared to the traditional 96 well individual format. Thus, using the 384 well approach we can have high resolution screening of 1263 individual peptides in 72 peptide pools generated from a 36x36 matrix, using 100 mL of blood readily available by venipuncture.

3. In 96-well ImmunoSpot® assays, a linear relationship is observed between the number of PBMC plated per well and the number of antigen-induced IFN-γ spots detected when the PBMC are plated between 100,000 -1,000,000 PBMC per well [18].

4. The assay can be miniaturized by using 384well plates, which reduces the number of PBMC to one third that of the original 96-well assay [18].

5. Using a 36 x 36 matrix, for example, permits high resolution testing of 1263 peptides with 100 mL of blood, see also Note 2.

6. ImmunoSpot® assays record the number of memory T cells that engage in cytokine production after recognition of their cognate antigen/peptide [18].

7. Typically, IFN-γ secretion by T cells is being measured, but the assay can be multiplexed to detect several cytokines simultaneously (dual-color enzymatic [23], and 4-color fluorescent [24]).

8. Media control wells (A1, A2) on all plates can be summed up as the negative control for each test subject. In addition, as < 90% of the peptides are likely not to induce SFU beyond the mean

and SD of the media control, all negative peptides can be used to establishing the background for each test subject. As positive control, the CERI peptide pool and CPI are recommended for wells A3 and A4, respectively [25].

9. SpotMap™ by CTL also provides barcode printing options for the plates. Bar code recognition not only permits automated plate identification by a suited reader, but also verifies the orientation of the plate.

10. The plate layout is provided to a peptide manufacturer to synthesize the peptide library according to this design into barcoded 96 (or 384) well plates: synchronizing plate layout with peptide synthesis, testing, and counting, and integration of the 96 (or 384) well-based assay results is into a database for analysis is essential for a streamlined and error-free work flow.

11: We recommend depositing 20 µg peptide per well in barcode-identified 96-well plates, that, when dissolved in 200 µL will yield a 100 µg/mL (100 x) stock solution. Smaller variation in peptide quantities/concentrations is unavoidable, and acceptable, as the dose response range of T cells to peptides typically accommodates such differences [17].

12: Such peptides cannot, and do not need to be purified: purified and unpurified peptides typically provide similar results, e.g., peptide **pp65(495-503)** in Figure 1 in [16]. This probably is because (a) the TCR of the T cell population primed *in vivo* with the relevant peptide X^1 will bind to peptide variant X^1 in the recall assay too, neglecting non-cross-reactive variants $X^{2\text{-}y}$, (b) while the concentration of X^1 will not be stringently defined among all variants $X^{1\text{-}y}$, the dose response characteristic of the recall response is lenient in a wide range of concentrations (see also Note 11).

13. We recommend dissolving the peptides a couple of days before the first test is to be done. Until then, store the peptides in the lyophilized powder form, protected from moisture and light, at -20°C. Transfer the plates to room temperature a couple of hours before dissolving them to allow peptides and the plates to come to room temperature.

14. For dissolving the peptides, we recommend a two-step process. In the first step, a 100 x master stock solution is created, and in the second, a 10 x master stock solution. For the first step, 100 µL of room temperature PBS containing 10% DMSO and 1% Penicillin/Streptomycin is added to each well of the original plate (that has been brought to room temperature, see Note 13). This should be done on the benchtop avoiding drafts (not under the laminar flow hood) and with a multichannel micropipettor by slow addition of the liquid to prevent dust-dissemination of the powder. Ideally, using a 96 well (or 384 well) pipettor, changing tips between each plate to avoid carryover of peptide. Sterile tips and fluids are to be used, with the antibiotics providing additional protection. The peptides are now at 200 µg/mL (200 x) and ready for color coding.

15. We recommend to color code the peptides by adding neutral Reagent Tracker™ (RT) Dyes [26] (CTL, Cat #: CTL-RT1-010, CTL-RT2-010, CTL-RT3-010, CTL-RT4-010) to the master plates in a patten that permits visual and/or reader-based quality control of peptide handling (See Figure 4). For this, RT Master Plates are prepared containing the arranged RT Dye solutions at a 200 x concentration (see Figure 4), dissolved in PBS with 1% Penicillin Streptomycin. Using a 96 (or 384) channel pipettor, 100 µL (50 µL) of each well of the RT Master Plate is now transferred to the 200 x Peptide Master Plate. The peptides are now color coded at 100 µg/mL, 100 x the concentration needed for the actual testing.

16. We recommend creating barcoded 10 x Peptide Master Plates, by diluting and aliquoting the above 100 x color-coded Peptide Master Plates into ten 10 x Peptide Master Plates. For a 96

well design, transfer 20 µL of the 100 x Peptide Master Plate into to barcoded plates containing 180 µL/well of CTL-Test™ supplemented with 1% Penicillin Streptomycin using a 96 well pipettor. For a 384 well design, transfer 10 µL of the 100 x Peptide Master Plate into to barcoded plates containing 90 µL/well of CTL-Test™ supplemented with 1% Penicillin Streptomycin using a 384 well pipettor. Check visually, or better yet, verify with CTL's Reagent Tracker the accuracy of peptide transfer and concentration. Seal plates with an adhesive foil which avoids both spilling and evaporation. Store plates to be used for testing within a week at 4°C, freeze and store remainder of 10 x Peptide Master Plates at -20°C.

17. We recommend using barcoded plates, and color-coded peptides (see Figure 4) for creation of all Peptide Master Plates, and also for the actual assay. In this way, peptide identity can be unambiguously tracked throughout the entire process.

18. For performing the actual high-throughput ImmunoSpot® assay, we recommend the following steps. Using a 96 (or 384) channel pipettor, first plate the freshly thawed PBMC at the desired cell number per well (see Comment 3 and 4), in 180 µL/well for 96 well plates, and 90 µL/well for 384 well plates. For thawing, follow the protocol detailed in [27]. Keep the cells in the incubator in the final test medium (CTL-Test™ supplemented with 1% Penicillin Streptomycin, and 1% fresh L-Glutamine) at ready-to-plate concentration in flasks with loosened lids until plating, and return the plates to the incubator as soon as the cells have been plated. Assuring that the cells are "happy" throughout testing is key to a successful T cell assay. Into such ImmunoSpot™ plates already containing the PBMC, add the peptides from the 10 x Peptide Master Plates (20 µL/well for 96 well assays, 10 µL/well for 384 well assays, with a 96 or 384 channel pipettor). After the transfer of the peptides, "tap" each plate to make sure the peptide and the cells are evenly distributed in the wells ("Tapping" is demonstrated in the video: https://www.youtube.com/watch?v=40CSxWQS1DA). Visual, or better yet, scanner-based

automated color verification, permits to rapidly verify whether the peptide transfer was qualitatively and quantitatively accurate (see Note 19). Handle plates one by one. Once a plate is done, place it immediately into the incubator. You may stack 3-4 plates on top of each other, but do not touch the plates during the following 24h of the T cell activation period, and avoid brisk opening/closing of the incubator door during this period, as that will result in displacement of the T cells on the membrane and thus interfere with their detection via their secretory footprint.

19. CTL's Reagent Tracker Software has been designed for rapid verification of color-coded reagents in 96 or 384 well format. For each well on a plate, the actually measured color and its density are automatically compared to a pre-assigned color template. In this way, it can be verified for each well whether (a) the right peptide was plated, (b) in the right concentration, (c) whether spilling occurred, and (d) the plate orientation is verified. This quality control scan can be done either right after the peptides had been added.

20. After the 24 h test period, the cells are typically discarded (however, they can also be rescued for subsequent re-testing, see Note 21), and the plate-bound cytokine footprints are visualized. All these steps can be conveniently done using automated plate washers (see also Note 29) and 96 or 384 well pipettors. At this time all plates can be processed in a single batch (unlike for the pipetting of the peptides and cells where one by one handling of plates is recommended, see Note 18).

21. T cells not only survive ImmunoSpot™ assays unharmed, but can be rescued for re-testing [28]. To do so, at the end of the 24h T cell activation culture, transfer the cells from the ImmunoSpot™ plates into regular 96 well tissue culture plates with a 96 multi-channel pipettor, as follows: the cells are in 200 µL/well. Adjust pipette volume to 150 µL/channel, and gently

aspirate and eject that volume 3 x to resuspend the cells in each well, while minimizing shear forces (see Note 28) and avoiding the touching of the membrane. Aspirate and transfer 150 µL of each well into the corresponding tissue culture plate. Change tips, readjust pipet volume to 100 µL per channel, add 100 µL fresh complete medium per well to the ImmunoSpot® plate – without adding IL-2, IL-15 or other T cell growth factors (see Note 22), aspirate 100 µL/well, and add it to the corresponding wells of the tissue culture plate. Progress with washing and developing the ImmunoSpot® plate (see Note 20), and continue to incubate the tissue culture plate until the results of the epitope scan results become available, one or two days later. Now you may retest cells originating for wells that scored positive. Such tests are best done 7-9 days after the initial ImmunoSpot® assay. Spin plates and discard supernatant by flipping. Wash plate 2x witch complete medium. Resuspend cells from the positive wells by gentle 2x pipetting, and split them into two ImmunoSpot® test wells, 100 µL each well. As antigen presenting cells (APC), add autologous PBMC that have been T cell depleted (e.g., by magnetic bead separation; no irradiation or mitomycin treatment of the PBMC needed); add 100,000 such PBMC/well, in 30 µL. Add 20 µL of the 10 x peptide for which the cells tested positive to one of the wells, to reproduce the previous result, and 20 µL of a negative peptide. Perform the ImmunoSpot® assay as before. Afterwards, the peptide-reactive T cells can be rescued again, and kept in culture following standard protocols to grow T cell lines, but here the addition of IL-2 is needed.

22. Ideal media for testing T cells are formulated to provide low background and high signal. CTL-Test™ is such a medium: it outperforms serum-containing media for primary T cell testing in PBMC (see Figure 6 in [29]), and hence we recommend it for high-throughput epitope mapping, but as with all synthetic media, it is unsuited for T cell culture beyond 10 days. For that, switching to media containing qualified serum is required.

23. The counting of the secretory footprints produced by individual peptide- activated T cells (spot forming units, SFU) per well is done in a fully automated manner by ImmunoSpot® Readers, whereby IntelliCount™ algorithms are used to identify “spots” based on size/density/shape criteria over background noise [30]. In this way, the number/frequency of peptide-specific T cells within all PBMC plated per well is established. All ImmunoSpot® Readers are suited to accomplish this task in a walk-away, high-throughput manner, with barcoded plates being fed by stackers/robotic arms, and the SFU counts automatically integrated into the Spot-Map™ software, or any other mainstream database that permits the alignment of the individual peptides’ identity and location in a particular well of a particular plate with the corresponding SFU counts. Thus, the evaluation of the results is fully automated. Custom solutions are available for those with ultra-high-throughput requirements exceeding the regular capacity of analyzing three hundred 96-well plates (27,600 peptide-induced responses plus controls) in approximately 5 hours of fully automated time, with all audit trails included.

24. PBMC can be cryopreserved such that they maintain full T cell functionality, for which it is essential, however, to add the DMSO-containing freezing solution at room temperature to the cells being at room temperature (not ice-cold freezing medium to ice cold cells!) [31]. As the use of suboptimal serum can increase the background in the T cell assay, it is recommended to freeze the cells under serum-free conditions, using, e.g., CTL-Cryo™ ABC Media Kit. Never chill the PBMC before freezing; keep them at room temperature at all times.

25. Freeze-thawing of cells inevitably causes a fraction of cells (typically about 20%) to die, and the DNA strands that such cells release can cause clumping in the thawed cell material. This cell clumping can be avoided by including the immunologically neutral endonuclease, benzonase. Ready to use benzonase-containing serum-free wash solutions are available: CTL Anti-Aggregate Wash™ 20x Solution.

26. If it will be more than 10 minutes before plating cells into the ImmunoSpot® assay, cell suspensions should be placed in the incubator with caps loosened to allow for adequate gas exchange.

27. Tap plate and place it into the incubator as soon as the peptides have been added. “Tapping” is demonstrated in the video: https://www.youtube.com/watch?v=40CSxWQS1DA).

28. PBMC cells are sensitive to shear forces. Resuspend and plate them gently. Avoid vortexing and vigorous pipetting with narrow orifice tips.

29. An automated plate washer may be used if the pin height is adjusted to avoid touching the PVDF membrane at the bottom of the plate. The automated washer should be programmed not to aspirate the final wash, as this will remain in the plate to avoid drying of the membrane before addition of subsequent development reagents.

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Conflicts of Interest

P.V.L. is Founder, President and CEO, A.A.L. the COO of CTL, a company that specializes in immune monitoring by ImmunoSpot®. D.R.R. is an employee of CTL.

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Figures:

Peptides Tested				Inidiviudal Subjets' CD8+ T Cell Response (SFU per 1,000,000 PBMC)									
Peptide Name	Sequence	Binding Score	Rank	ID 1	ID 2	ID 3	ID 4	ID 5	ID 6	ID 7	ID 8	ID 9	ID 10
pp65:018-026	ISGHVLKAV	7.9	64	0	7	7	0	23	3	240	37	0	20
pp65:014-022	VLGPISGHV	0.23	9	3	3	50	10	17	0	10	80	27	27
pp65:030-038	GDTPVLPHE	50	346	0	7	10	3	7	7	17	30	3	107
pp65:040-048	RLLQTGIHV	0.11	3	0	3	0	23	10	0	17	43	7	3
pp65:065-073	STPCHRGDN	97	535	53	0	0	147	0	3	3	17	0	3
pp65:070-078	RGDNQLQVQ	56	375	7	0	7	7	0	7	280	93	7	17
pp65:095-103	HNPTGRSIC	75	460	50	0	67	0	0	3	7	33	3	3
pp65:097-105	PTGRSICPS	86	507	0	0	137	3	70	0	30	17	0	7
pp65:103-121	CPSQEPMSI	27	216	50	0	20	7	3	0	17	37	7	23
pp65:106-114	QEPMSIYVY	32	239	47	0	53	17	0	0	7	43	3	10
pp65:107-115	EPMSIYVYA	16	126	0	0	57	7	7	0	7	1343	3	3
pp65:114-121	YALPLKMLN	39	281	73	3	23	0	10	7	77	47	0	10
pp65:115-123	ALPLKMLNI	0.82	17	43	0	17	23	7	0	20	73	20	7
pp65:116-124	LPLKMLNIP	56	376	237	0	23	47	7	10	17	60	17	7
pp65:119-127	KMLNIPSIN	25	202	17	0	20	340	7	3	70	33	0	0
pp65:120-128	MLNIPSINV	0.15	4	27	0	0	7	7	0	30	50	10	27
pp65:139-148	HRHLPVADA	45	320	43	3	3	60	3	3	20	30	17	10
pp65:141-149	HLPVADAVI	3.3	36	23	0	3	0	87	0	17	27	0	10
pp65:142-150	LPVADAVIH	68	427	37	0	7	33	0	0	0	20	33	3
pp65:144-152	VADAVIHAS	12	92	3	7	17	0	147	3	7	10	10	20
pp65:149-157	IHASGKQMW	25	205	0	0	1750	3	7	0	3	7	7	7
pp65:151-158	ASGKQMWQA	9.2	74	67	0	7	10	0	3	23	20	3	0
pp65:152-160	SGKQMWQAR	50	347	77	0	30	23	0	3	33	30	10	3
pp65:155-163	QMWQARLTV	0.38	12	3	3	23	3	33	7	110	43	17	0
pp65:175-183	WKEPDVYYT	37	274	3	0	7	0	480	0	3	23	0	3
pp65:188-196	FPTKDVALR	30	234	3	3	3	17	20	3	2317	10	43	3
pp65:203-211	ELVCSMENT	20	163	393	0	0	7	3	3	70	10	23	3
pp65:208-216	MENTRATKM	26	210	3	3	237	23	17	3	0	47	37	10
pp65:218-226	VIGDQYVKV	0.33	10	0	0	23	7	0	0	20	83	17	0
pp65:221-229	DQYVKVYLE	29	229	3	3	23	3	253	0	0	33	20	0
pp65:228-236	LESFCEDVP	95	527	0	0	7	20	33	3	3	7	0	17
pp65:250-258	VEEDLTMTR	24	199	10	0	10	20	43	10	7	7	7	3
pp65:251-259	EEDLTMTRN	85	499	0	3	3	7	3	7	357	3	10	7
pp65:262-270	PFMRPHERN	97	536	0	3	537	17	0	7	20	10	30	3
pp65:267-275	HERNGFTVL	20	164	0	0	10	0	0	0	20	7	0	153
pp65:270-278	NGFTVLCPK	66	420	0	7	33	0	3	0	17	30	23	1033
pp65:273-281	TVLCPKNMI	9	71	3	0	30	207	0	7	23	7	10	7
pp65:284-292	PGKISHIML	48	336	37	0	0	37	7	23	20	23	33	60
pp65:320-328	LMNGQQIFL	0.17	6	47	7	33	57	3	0	70	7	3	70
pp65:324-332	QQIFLEVQA	6.1	54	1143	0	17	10	10	0	20	17	3	27
pp65:325-333	QIFLEVQAI	2.1	32	1327	3	20	53	17	3	3	43	0	23
pp65:328-336	LEVQAIRET	44	316	0	17	2700	23	3	3	23	33	17	70
pp65:340-348	RQYDPVAAL	0.15	5	20	23	17	20	0	7	3	7	17	70
pp65:347-355	ALFFFDIDL	0.17	7	0	0	33	77	0	0	10	47	3	10
pp65:349-357	FFFDIDLLL	0.19	8	3	3	43	50	3	0	3	30	3	20
pp65:390-398	EGAAQGDDD	100	546	0	0	17	187	0	0	23	43	7	20
pp65:395-403	GDDDVWTSG	60	391	7	0	10	33	0	0	47	327	3	7
pp65:417-425	TPRVTGGGA	60	392	0	0	10	107	0	3	33	7	1860	7
pp65:418-426	PRVTGGGAM	51	359	3	0	20	20	0	0	20	37	640	0
pp65:430-438	STSAGRKRK	52	363	10	3	0	297	3	0	113	60	37	23
pp65:431-439	TSAGRKRKS	79	477	0	0	27	30	0	3	307	57	10	7
pp65:465-473	EEDTDEDSD	95	531	0	3	37	180	3	3	17	20	23	23
pp65:482-490	FTWPPWQAG	13	110	0	70	3	47	3	3	10	10	33	10
pp65:492-500	LARNLVPMV	7.5	62	70	7	17	20	0	0	7	3	17	0
pp65:495-503	NLVPMVATV	0.06	1	200	1010	3	333	323	493	957	2247	47	1060
pp65:503-511	VQGQNLKYQ	50	351	7	3	1707	3	0	3	17	10	17	0
pp65:511-519	QEFFWDAND	83	496	7	3	20	30	0	0	27	57	3	317
pp65:512-520	EFFWDANDI	16	132	0	93	17	33	3	3	27	43	57	203
pp65:513-521	FFWDANDIY	4.2	42	3	83	17	20	0	3	7	27	27	333
pp65:514-522	FWDANDIYR	15	122	0	7	7	3	0	3	33	43	33	147
pp65:521-529	YRIFAELEG	42	308	7	3	267	17	0	0	93	23	3	17
pp65:522-530	RIFAELEGV	0.07	2	17	20	3	30	0	27	17	37	27	33
pp65:524-532	FAELEGVWQ	42	309	7	53	20	27	3	23	80	27	37	20
pp65:544-552	QDALPGPCI	32	244	7	20	17	10	50	17	43	17	7	7
		Negative Controls and Cut Off Values For Response Categories	□	3	3	14	13	13	6	22	28	18	11
			σ	3	4	12	15	2	8	19	19	12	9
			□*3σ	13	15	50	57	18	29	78	84	55	38
			□*5σ	19	24	74	86	22	45	115	121	80	56
			□*10σ	36	46	133	159	30	84	208	214	142	101
			>334 SFU	>334	>334	>334	>334 SFU	>334	>334	>334	>334 SFU	>334	>334

Figure 1: Example of aleatory epitope recognition by CD8+ T cells in HLA allele matched individuals*. Ten HCMV-positive individuals, all expressing the HLA-A02:01 allele, were tested for T cell reactivity against 553 peptides that systematically cover the sequence of the HCMV pp65 protein (as illustrated in Figure 1). The numbers of T cells responding by IFN-γ production normalized to 1,000,000 PBMC tested by ImmunoSpot® are shown. High frequency ("dominant") recall responses are highlighted in red, intermediate "subdominant" responses in orange, statistically significant but weak "cryptic" responses in yellow. Only the peptides that elicited a positive response in at least one of the test subjects are listed. Peptides with predicted binding for the shared HLA-A02:01 allele are highlighted in green, with the binding score and rank specified according to the predicted binding. Of note: peptide 495-503 is the only one that is co-dominant in several (5 of 10) of these subjects, being just one of several immune dominant epitopes in most individuals. This 495-503 peptide might be unique, however: while it ranks highest for predicted HLA-A02:01 binding, it also ranks highest for predicted binding for many other alleles expressed by these individuals. The data are adapted from, and are fully described in [9].*

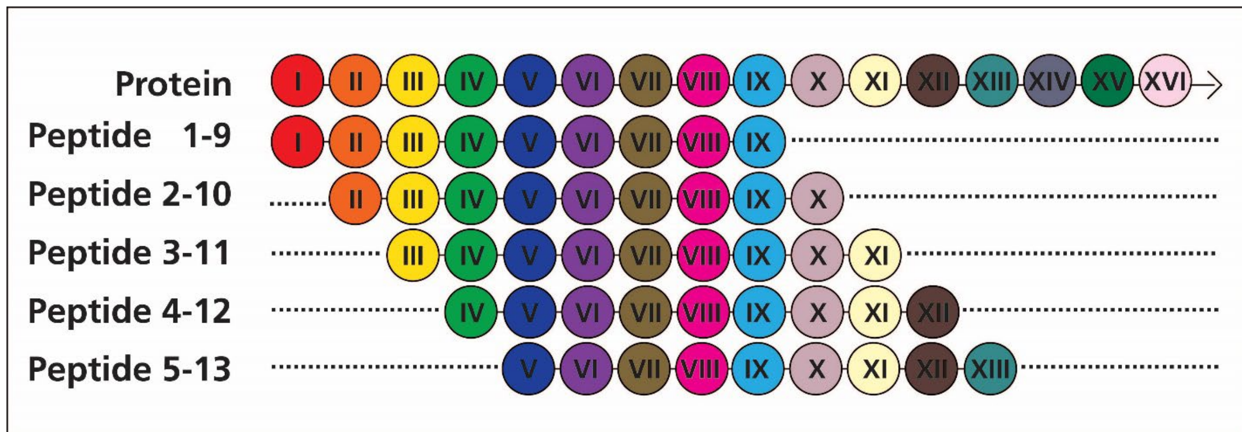

Figure 2: Comprehensive epitope mapping illustrated*. To test for CD8+ T cell epitope recognition a library of 9-mer peptides is synthesized and tested – this peptide length is chosen because MHC/HLA Class I molecules' peptide binding groove is closed on both ends, permitting it to bind peptides 8-10 amino acids long [22]. The individual peptides walk the protein's amino acid sequence amino acid by amino acid, thus providing complete coverage for any possible epitope.*

Peptide Pool	PP 1	PP 2	PP 3	PP 4	PP 5	PP 6	PP 7	PP 8	PP 9	PP 10
PP 37	1	2	3	4	5	6	7	8	9	10
PP 38	37	38	39	40	41	42	43	44	45	46
PP 39	73	74	75	76	77	78	79	80	81	82
PP 40	109	110	111	112	113	114	115	116	117	118
PP 41	145	146	147	148	149	150	151	152	153	154
PP 42	181	182	183	184	185	186	187	188	189	190
PP 43	217	218	219	220	221	222	223	224	225	226
PP 44	253	254	255	256	257	258	259	260	261	262
PP 45	289	290	291	292	293	294	295	296	297	298
PP 46	325	326	327	328	329	330	331	332	333	334

Figure 3: Testing peptides pooled into matrix format permits identification of the individual peptides that have elicited T cell responses. *A 10 x 10 subsection of a 36 x 36 matrix is shown as an example. The peptide pools specified on the horizontal axis contain the individual peptides listed in the corresponding columns, while the peptide pools specified on the vertical axis contain the individual peptides listed in the corresponding lines. As the peptide pools overlap in individual peptides only, that peptide is identified as positive if the T cells respond to the corresponding two pools. For a detailed description of the matrix testing approach see [32].*

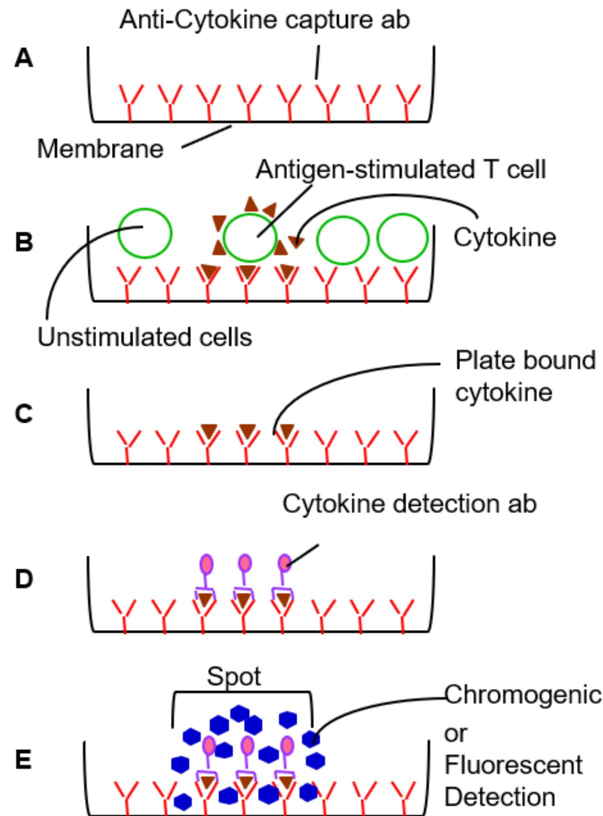

Figure 4: Illustration of ImmunoSpot® testing for revealing antigens/peptides that have induced T cell responses in vivo*. (A) A special PVDF membrane-bottomed 96 (or 384 well) plate is coated with a capture antibody (or, in the case of multiplexed assays, several capture antibodies) specific for the cytokine(s) to be detected. Onto this sensitized membrane, the PBMC are plated in numbers to form a coherent monolayer [18], and the antigen/peptide is added. During a subsequent 24h cell culture period, the antigen/peptide-specific T cells become activated and engage in cytokine secretion – this cytokine is captured on the membrane around each secreting T cell, being retained as a secretory footprint on the membrane after the cells are removed (C), which then is detected by addition of cytokine-specific detection antibody (D). (E) The plate-bound detection antibody is then visualized either by an enzymatic reaction creating a precipitating substrate (single- or double-color enzymatic assays that can be analyzed under white light, [23]), or by multicolor fluorescence [24].*

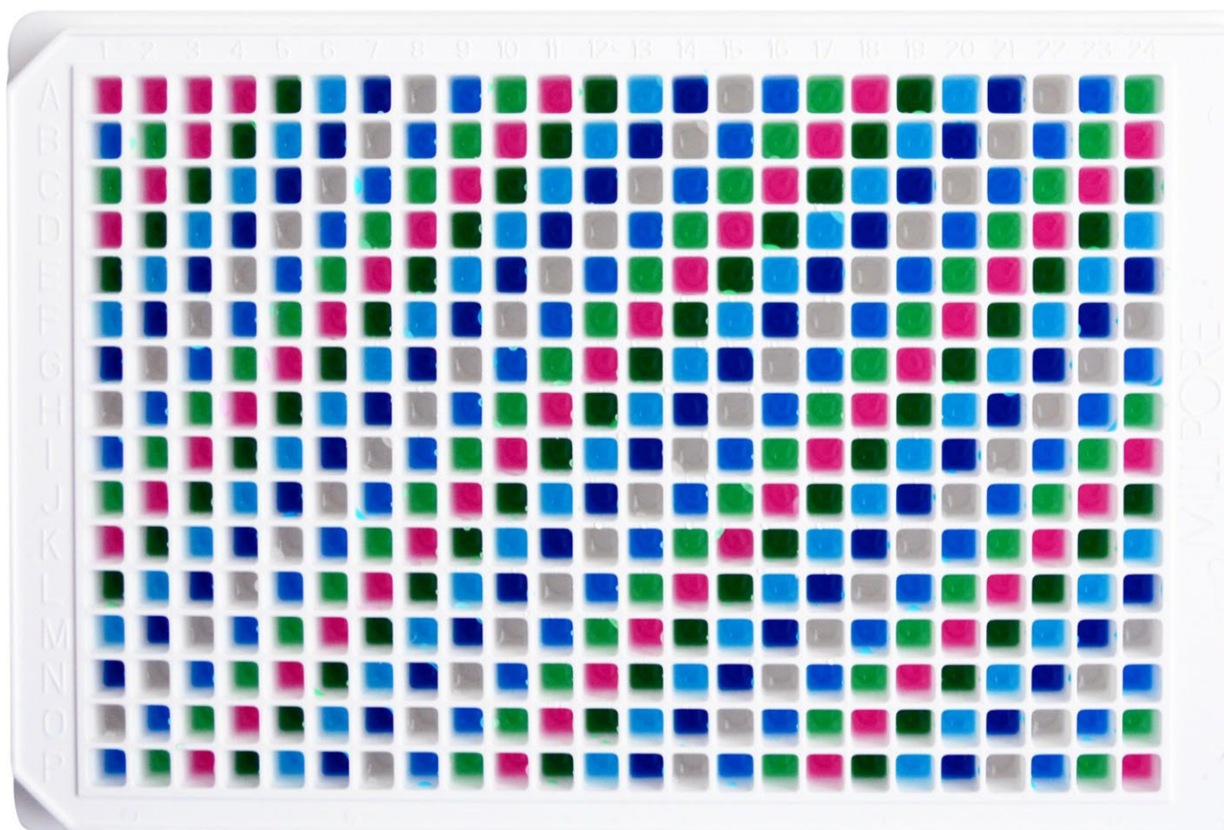

Figure 5: Illustration of the Reagent Tracker™ technique for assurance of peptide identity, purity, and concentration during high-throughput peptide testing*. A 384 well 100 x Master Peptide Plate is shown. Biologically neutral Reagent Tracker™ dyes [26] are admixed to the individual peptides on the master plates to obtain a distinct pattern. Wells A1-A4 are reserved for negative and positive controls. When liquid is transferred with multi-channel pipettors successfully to the corresponding 10 X Master Peptide Plates, and then to the actual test plates, the pattern of the distinct colors is maintained, verifying the transfer, and the lack of spill-overs. Moreover, the dilution of the dye permits to measure whether the planned volume has indeed been transferred. Using CTL's Reagent Tracker™ Suite, the actually measured and expected result can be compared in a fast, automated fashion.*